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Importance of the Intestinal Inflammatory Reaction in Salmonella-Mediated Intestinal Secretion

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The ability of *Salmonella typhimurium* to invade the intestinal epithelium is essential to the pathogenesis of salmonella-induced intestinal secretion. This invasion is accompanied by an intense acute inflammatory reaction. The present study tests the hypothesis that the acute inflammatory reaction may have a role in the pathogenesis of salmonella-induced secretion. Two groups of rabbits infected with *S. typhimurium* were studied: normal animals and animals pretreated with nitrogen mustard. Nitrogen mustard depletes the polymorphonuclear leukocyte pool and thereby prevents the formation of an acute inflammatory reaction. In vivo ligated ileal loops were constructed and infected 72 h after nitrogen mustard administration when polymorphonuclear leukocytes were undetectable. Nitrogen mustard treatment markedly inhibited salmonella-induced secretion. Ileal histology in normal animals infected with *S. typhimurium* revealed an intense acute inflammatory reaction, while in animals pretreated with nitrogen mustard only a rare polymorphonuclear leukocyte was seen. The antisecretory effect of nitrogen mustard was not merely a nonspecific effect since nitrogen mustard treatment did not inhibit cholera toxin-induced secretion and did not alter either ileal morphology nor the activities of various intestinal enzymes in normal animals. Nitrogen mustard also did not alter the virulence of the inoculated *S. typhimurium*. These data suggest that the mucosal inflammatory reaction induced by salmonella invasion may be important to the pathogenesis of the salmonella secretory process. The mechanism by which the inflammatory reaction stimulates secretion is not known.

Infection of the gastrointestinal tract with *Salmonella typhimurium* frequently results in intestinal fluid secretion and diarrhea. Although the mechanisms by which this infection causes fluid secretion are incompletely understood (9), we have previously shown that the ability of *S. typhimurium* to invade the intestinal epithelium is essential to the pathogenesis of intestinal secretion (8, 12, 17). Mucosal invasion by this organism is regularly accompanied by an intense acute inflammatory reaction (8, 12, 17). Therefore, we hypothesized that this inflammatory reaction may have a role in the pathogenesis of salmonella-induced intestinal fluid secretion. The purpose of this study is to test this hypothesis. Our rationale was as follows: if an acute inflammatory reaction within the intestinal mucosa is important to salmonella-induced secretion, abolition of the mucosal inflammatory reaction should result in the inhibition of salmonella-induced secretion.

MATERIALS AND METHODS

Bacterial strain. *S. typhimurium* TML, isolated from an adult with severe cholera-like diarrhea (10, 11), is a virulent strain for humans and animals and is described in previous publications (8, 10-15, 17-18, 22, 30). The strain was maintained in the lyophilized state, and a new ampoule was used for each experiment. Organisms were grown overnight in brain-heart-infusion broth, harvested by centrifugation, and washed with and suspended in isotonic saline to give the desired concentration of organisms.

Animal model. The in vivo-ligated rabbit ileal-loop model was used as previously described (8, 12, 13, 15, 17, 18). New Zealand albino rabbits weighing 2 to 3 kg were housed individually, and food was withheld from them for 48 h before the study. Three loops, ca. 15 cm in length, were constructed in each animal: an experimental loop inoculated with live organisms or cholera toxin, a negative control loop inoculated with saline, and an interposed uninoculated loop. Each experimental loop was inoculated with a total volume of 1.0 ml containing either 10^9 *S. typhimurium* or 0.5 μg of purified cholera toxin (Schwarz/Mann, Orangeburg, N.Y.). Animals were sacrificed 18 h after loop inoculation, fluid secretion was measured, and mucosal homogenates assayed for maltase, sucrase, and adenylate cyclase activities and protein concentration. Sections of all control and experimental loops were fixed in Formalin, stained with hematoxylin and eosin, coded, and studied by light microscopy.

Enzyme assays. Adenylate cyclase activity was measured in whole mucosal homogenates by the method of Krishna et al. (24) as described previously (3, 13). Mucosa was obtained by scraping with a glass slide and was homogenized in cold 75 mM tris(hydroxymethyl)aminomethane buffer (pH 7.6) and assayed in triplicate. Activity was expressed as picomoles of cyclic AMP formed per milligram of protein per 10 min.

Another portion of the mucosal scrapings was homogenized in cold saline and assayed for sucrase and maltase activities by the method of Dahlquist (5) as described previously (16). Activity was expressed as micromoles of substrate hydrolyzed per minute per gram of mucosal protein.

Mucosal protein concentrations for the various assays were measured by the method of Lowry et al. (25) using bovine serum albumin as a standard.

Inhibition of the inflammatory response. Our approach was to use nitrogen mustard (NM, Mustargen, Merck Sharp & Dohme, West Point, Pa.) to deplete the animal of polymorphonuclear leukocytes (PMN) as described by Humphrey (21) and by Cochrane et al. (4). It was reasoned that depletion of PMN by NM would prevent the occurrence of an acute inflammatory reaction subsequent to salmonella infection. NM, 1.75 mg/kg, was injected intravenously, and its effect was monitored with sequential complete blood counts. This dose was chosen because it consistently caused a fall in PMN of sufficient duration while resulting in no obvious untoward effects on the animals.

Data presentation and statistical methods. All data are presented as mean ± standard error of the mean and evaluated statistically by the unpaired *t* test (33).

RESULTS

Effect of NM on circulating leukocyte (WBC) count. The effect of NM on total WBC count and on number of PMN in five normal rabbits is shown in Fig. 1. NM caused a prompt fall in the number of circulating PMN. At 48 h, there were a few remaining PMN, but at 72 h, the number of PMN was zero and remained undetectable for at least the next 24 to 48 h. The total WBC exhibited a similar pattern reaching a plateau between 48 to 96 h. No changes in hemoglobin concentration or in hematocrit were noted.

Experimental groups and experimental design. Two groups of animals were studied—a group inoculated with *S. typhimurium* and a group inoculated with cholera toxin. Each of these groups was divided into a group of animals pretreated with NM and a group of normal animals who were not pretreated.

NM was administered, and 72 h later when the PMN count was zero, intestinal loops were constructed. Eighteen hours later, during which time the PMN count remained at zero, the animals were sacrificed.

Effect of NM on salmonella-infected animals. The effect of NM administration on salmonella-induced secretion is shown in Fig. 2. In animals infected with *S. typhimurium* but untreated with NM, substantial ileal secretion was evident, 1.12 ± 0.18 ml/cm (mean ± standard error). In contrast, in animals infected with *S. typhimurium* but pretreated with NM, ileal secretion was markedly inhibited, i.e., 0.38 ± 0.16 ml/cm. This difference was statistically significant, $P < 0.01$.

The effect of NM on the hematological parameters of salmonella-infected animals is shown in Fig. 3. Normal rabbits infected with *S. typhimurium* demonstrated a prompt rise in both total WBC and PMN counts. In contrast, salmonella-infected animals who had been pretreated with NM demonstrated no change in total WBC count, while circulating PMN remained undetectable.

The lack of an ileal mucosal acute inflammatory reaction in salmonella-infected animals pretreated with NM was documented by histological examination. Figure 4 contrasts the ileal morphology of an animal infected with *S. typhimu-*

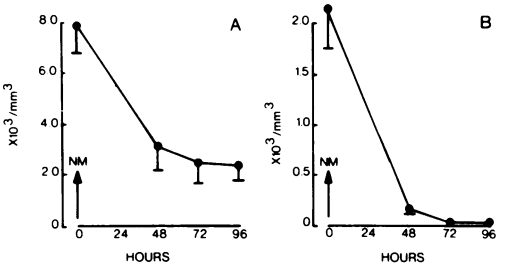

FIG. 1. *Effect of NM on total WBC count (A) and number of PMN (B) in blood of normal rabbits. Each point is the mean ± 1 standard error of the mean of five normal rabbits.*

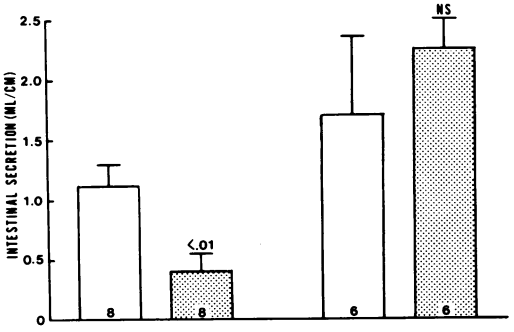

FIG. 2. *Effect of NM on salmonella-mediated (left-hand bars) and cholera toxin-induced (right-hand bars) ileal secretion. ☐, Control animals; ☐, NM animals. Numbers in bars signify number of animals in each group. NS, Not significant; <0.01 = P < 0.01.*

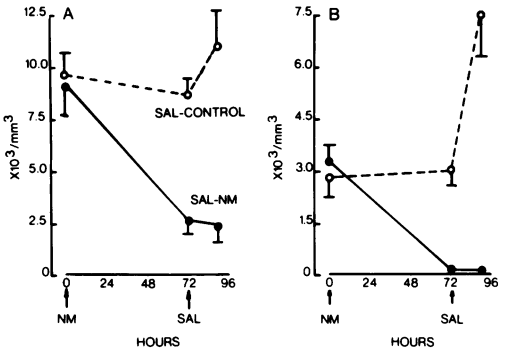

FIG. 3. *Effect of NM on total WBC (A) and number of PMN (B) in blood of salmonella-infected rabbits. First arrow (NM), time of NM administration. Second arrow (SAL), time of inoculation with S. typhimurium. SAL-control signifies normal animals infected with S. typhimurium, and SAL-NM signifies animals treated with NM before infection. Each point is the mean ± 1 standard error of the mean of eight rabbits.*

rium but untreated with NM (A) with that from an animal infected with *S. typhimurium* but pretreated with NM (B). In the former, the villi are blunted and swollen and intensely infiltrated with PMN. The superficial epithelial cells are disordered and flattened, approaching a cuboidal epithelium. In contrast, with NM pretreatment only a rare mucosal PMN was seen, although there were other mild abnormalities consisting of shortened villi, absence of goblet cell mucus, and an increased number of intraepithelial lymphocytes.

Effect of NM on animals inoculated with cholera toxin. To examine whether the antisecretory effect of NM was merely a nonspecific effect, ileal fluid secretion in response to cholera toxin was compared in normal and NM-pretreated animals. This control was chosen because the secretion induced by cholera toxin is not associated with a mucosal inflammatory reaction (7, 28) but occurs by activation of the epithelial adenylate cyclase-cyclic AMP system (1).

The effect of NM administration on cholera toxin-induced ileal secretion is shown in Fig. 2. In animals exposed to cholera toxin alone, fluid secretion of 1.67 ± 0.65 ml/cm was seen. In animals pretreated with NM and then exposed to cholera toxin, NM had no significant effect on the magnitude of cholera toxin-induced secretion, i.e., 2.27 ± 0.25 ml/cm.

The effect of NM on the hematological parameters of cholera toxin-exposed animals is shown in Fig. 5. Neither normal rabbits nor rabbits pretreated with NM demonstrated any change in total WBC count or in number of circulating PMN after exposure to cholera toxin. In animals pretreated with NM, the number of PMN was zero at the time of ileal-loop inoculation and remained undetectable for the duration of the experimental loop inoculation period.

Histological examination of ileal loops from both these animal groups was normal.

Effect of NM on the intestine and on *S. typhimurium*. Because NM may have effects on tissues other than PMN, attempts were made to document whether the dose of NM utilized had toxic effects on the intestine or on the *S. typhimurium* organism. Figure 6 illustrates the light microscopic morphology of the normal rabbit ileum and compares it with the ileum of an animal 72 h after NM administration. There are no morphological differences.

Table 1 presents data comparing the activities of various intestinal enzymes in normal and NM-treated animals. There are no significant differences between these two animal groups in the brush border enzymes sucrase and maltase or in the basolateral membrane enzyme adenylate cyclase.

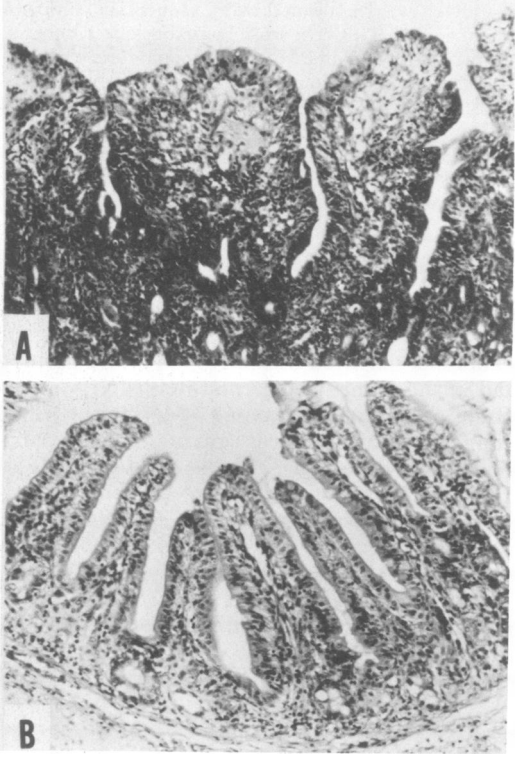

FIG. 4. *Comparison of ileal morphology of (A) rabbit infected with S. typhimurium but untreated with NM and (B) rabbit infected with S. typhimurium and pretreated with NM. Hematoxylin-eosin stain. ×125.*

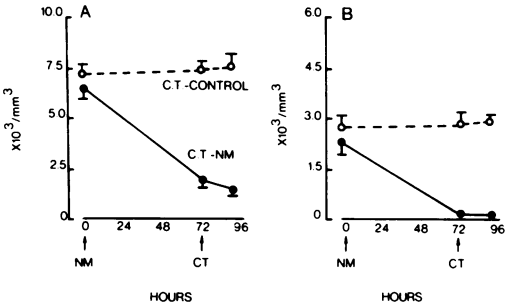

FIG. 5. *Effect of NM on total WBC (A) and number of PMN (B) in blood of cholera toxin-exposed rabbits. First arrow (NM), time of NM administration. Second arrow (CT), time of inoculation with cholera toxin. C.T.-control signifies normal animals given cholera toxin. C.T.-NM signifies animals treated with NM before cholera toxin administration. Each point is the mean ± 1 standard error of the mean of six rabbits.*

In addition, no deleterious effects of NM on *S. typhimurium* were apparent. Injected organisms proliferated to the same extent within ileal loops of normal or NM-treated animals; organisms in NM animals invaded equally well as evidenced by similar frequency of positive liver and spleen cultures between the two animal groups; and *S. typhimurium* isolated from NM-treated animals regularly caused ileal secretion and disseminated to liver and spleen when subsequently inoculated into normal rabbits.

However, it is possible that NM treatment altered host defenses in such a way that the distribution and multiplication of invading organisms within the intestinal mucosa is different from that of normal animals. While this possibility cannot be definitively excluded, examination of Giemsa-stained sections of ileal mucosa from the two animal groups revealed no gross difference in number or distribution of invading organisms.

DISCUSSION

The purpose of this study was to test the hypothesis that the acute inflammatory reaction of the gastrointestinal mucosa associated with salmonella invasion plays a role in the pathogenesis of salmonella-induced ileal secretion. The results of the present study demonstrate that when the inflammatory reaction in salmonella-infected rabbits is abolished by NM treatment, intestinal secretion is markedly inhibited. Thus, these findings support the proposed hypothesis.

This conclusion is based upon the assumption that the expected inflammatory reaction attendant upon salmonella infection was indeed prevented by NM treatment. Several lines of evidence document the absence of the mucosal inflammatory reaction. First, salmonella-infected rabbits pretreated with NM had no detectable circulating PMN both at the beginning and end of the infection period. Second, unlike normal animals infected with *S. typhimurium*, who demonstrated a marked rise in number of PMN during infection, in animals pretreated

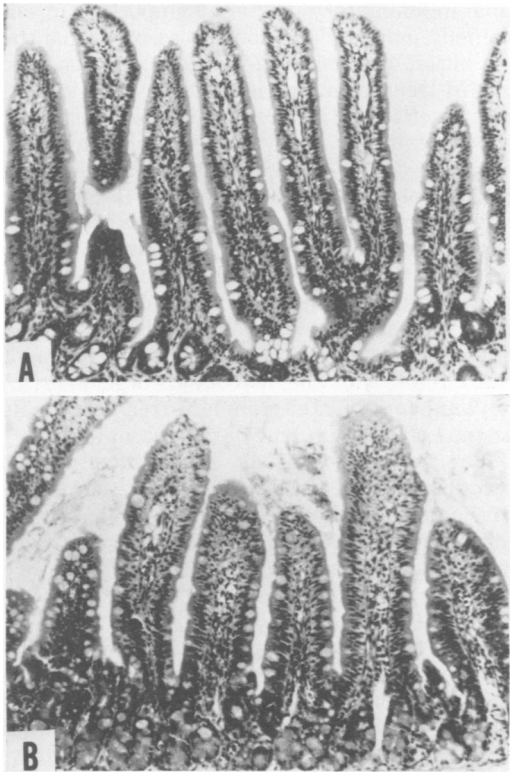

FIG. 6. *Comparison of normal rabbit ileum (A) and ileum of NM-treated rabbit (B). Hematoxylin-eosin stain. ×125.*

TABLE 1. *Effect of NM on intestinal enzyme activity*[a]

Animals	Sucrase[b]	Maltase[b]	Adenylate cyclase[c]
Control	44.8 ± 6.8 (8)	112 ± 17 (8)	287 ± 31 (18)
NM[d]	43.1 ± 8.5 (5)	92 ± 16 (5)	282 ± 34 (7)

[a] Mean ± 1 standard error. Numbers in parentheses represent the number of animals studied. There are no significant differences between groups.

[b] Micromoles of substrate hydrolyzed per minute per gram of protein.

[c] Picomoles of cyclic AMP formed per 10 min per milligram of protein.

[d] Seventy-two hours after NM administration (1.75 mg/kg).

with NM, PMN remained undetectable. Last, salmonella-infected rabbits pretreated with NM were demonstrated by light microscopy to have only a rare PMN within the mucosa compared to the intense infiltration with PMN seen in salmonella-infected rabbits not pretreated with NM.

Furthermore, the conclusion that the intestinal inflammatory reaction is important in salmonella-induced secretion also assumes that the inhibition of salmonella-induced secretion seen in NM-treated animals is due to the anti-inflammatory effects of NM rather than to nonspecific effects on the intestine or on the infecting organism. We do not believe the inhibitory effects of NM can be ascribed to nonspecific effects on the intestine since, at the dose used, NM did not alter intestinal morphology, did not alter either brush border or basolateral membrane enzyme activity, and did not inhibit cholera toxin-induced secretion. Nor can the inhibitory effects of NM be explained by induced alterations of the virulence of the inoculated organisms. *S. typhimurium* isolated from NM-treated animals seemed equally virulent to the parent strain.

The importance of the acute inflammatory reaction to salmonella-induced secretion is further underscored by the fact that the antisecretory effect of NM was confined to a secretory process associated with an acute inflammatory reaction, i.e., salmonella infection. NM treatment did not inhibit cholera toxin-induced secretion, a process not associated with mucosal inflammation but one that requires the biochemical activation of the intestinal adenylate cyclase-cyclic AMP system (1). This differential effect suggests that the pathogenesis of salmonella-induced secretion differs from the pathogenesis of cholera toxin-induced secretion (9).

It should be mentioned that we have previously described strains of *S. typhimurium* that invade the intestinal mucosa and cause an acute inflammatory reaction, but do not evoke fluid secretion (12). We do not believe these previous observations are contrary to our present hypothesis since these strains result in a quantitatively more modest acute inflammatory reaction than the strain (TML) used in the present study. It is possible that the magnitude of the acute inflammatory reaction must be of a certain threshhold level before intestinal secretion occurs.

The mechanism whereby acute inflammatory reactions within the gut might stimulate intestinal secretion is important for not only may it apply to salmonellosis but to other conditions associated with acute inflammation in which fluid secretion occurs, e.g., shigellosis (23, 31), idiopathic ulcerative colitis (6, 19), and parasitic infections (G. E. Whalen, J. Hessel, and G. Castro, Clin. Res. **26**:326A, 1978).

Unfortunately, the mechanism whereby the acute inflammatory reaction induced by salmonella infection, or any acute inflammatory disorder of the intestine, stimulates intestinal secretion is unknown. In the case of salmonellosis, this cannot be attributed to alterations in intestinal permeability because we have demonstrated previously, in both the rabbit and rhesus monkey models of salmonellosis, that transmucosal permeability is not altered (15, 22). Several other possibilities should be mentioned. It is possible that the inflammatory reaction causes the release of local hormones, e.g., vasoactive intestinal peptide, which have been shown to cause intestinal secretion (2, 32).

Indomethacin, a nonsteroidal anti-inflammatory agent and an inhibitor of prostaglandin synthesis, also inhibits salmonella-induced secretion (13–14, 18). Because salmonella infection of the intestine results in an intense acute inflammatory reaction (8, 12, 17) and acute inflammatory reactions elsewhere result in the synthesis and release of prostaglandins (20, 34) and prostaglandins induce intestinal secretion (26–27, 29), it is possible that prostaglandins released by the inflammatory process induce intestinal secretion. This hypothesis is speculative at present, although it is consistent with our observations that abolition of the inflammatory reaction markedly inhibits salmonella-induced secretion. We are currently further testing this hypothesis.

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